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RNA adenosine deaminase ADAR1 deficiency leads to increased activation of protein kinase PKR and reduced vesicular stomatitis virus growth following interferon treatment

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ABSTRACT

Two size forms of ADAR1 adenosine deaminase are known, one constitutively expressed (p110) and the other interferon (IFN)-induced (p150). To test the role of ADAR1 in viral infection, HeLa cells with ADAR1 stably knocked down and 293 cells overexpressing ADAR1 were utilized. Overexpression of p150 ADAR1 had no significant effect on the yield of vesicular stomatitis virus. Likewise, reduction of p110 and p150 ADAR1 proteins to less than ~10 to 15% of parental levels (ADAR1-deficient) had no significant effect on VSV growth in the absence of IFN treatment. However, inhibition of virus growth following IFN treatment was ~1 $\log_{10}$ further reduced compared to ADAR1-sufficient cells. The level of phosphorylated protein kinase PKR was increased in ADAR1-deficient cells compared to ADAR1-sufficient cells following IFN treatment, regardless of viral infection. These results suggest that ADAR1 suppresses activation of PKR and inhibition of VSV growth in response to IFN treatment.

Introduction

Adenosine deaminase acting on RNA (ADAR1) is an RNA editing enzyme that catalyzes the C-6 deamination of adenosine (A) to generate inosine (I) in RNA substrates that possess double-stranded RNA character (Samuel, 2001; Bass, 2002; Valente and Nishikura, 2005; Toth et al., 2006). A-to-I editing is of broad physiologic significance, because I is recognized as G instead of A by ribosomes during translational decoding of mRNA and also by polymerases during RNA-dependent transcription (Bass, 2002; Toth et al., 2006). Such substitution A-to-I editing is seen in both cellular and viral RNAs. The deamination editing can be site-selective, occurring at one or a few A's, thereby generating protein products with altered function because of selective amino acid substitutions arising from the substitution of A with I (G). Among the best characterized examples of selective editing specified by imperfect duplex RNA structures are the hepatitis delta virus antigenome RNA (Luo et al., 1990; Jayan and Casey, 2002) and the cellular pre-mRNAs for the L-glutamate (GluR) and serotonin-2c (5-HT_{2c}) receptors in the nervous system (Higuchi et al., 1993; Liu and Samuel, 1999; Liu et al., 1999; Maas et al., 2001; Seeburg and Hartner, 2003).

DsRNA-specific deamination by ADAR1 also can occur at multiple sites within perfect duplex RNA substrates (Liu and Samuel, 1996; Kumar and Carmichael, 1997; Liu et al., 2000). Indeed, dsRNA-specific adenosine deaminase enzymatic activity was first described as a developmentally regulated dsRNA duplex-unwinding activity in *Xenopus* oocytes (Rebagliati and Melton, 1987; Bass and Weintraub, 1988). But now it is recognized that, rather than unwinding duplex dsRNA to separate strands, the RNA becomes more single-stranded in character because stable A:U base pairs are changed to less stable I:U base pairs (Bass and Weintraub, 1988; Wagner et al., 1989).

The *ADAR1* gene is single copy, ~40-kbp with 17 exons, and maps to human chromosome 1q21 (Weier et al., 1995; Liu et al., 1997). ADAR1 is interferon-inducible (Patterson et al., 1995; George and Samuel, 1999; George et al., 2008). Two size forms of the ADAR1 protein are known, an IFN-inducible protein of ~150-kDa designated p150 that is found in both the cytoplasm and nucleus, and a constitutively expressed protein of ~110-kDa designated p110 that is predominantly if not exclusively a nuclear protein (Patterson and Samuel, 1995; Toth et al., 2006). At least three alternative promoters, one of which possesses an ISRE element and is IFN-inducible, together with alternative splicing, drive the expression of *ADAR1* transcripts with alternative exon 1 structures (George and Samuel, 1999; Kawakubo and Samuel, 2000; George et al., 2005, 2008). Translation initiation of the IFN-inducible 1200 amino acid protein (p150) begins at AUG1 present in the alternative exon 1A at the 5′-termini of IFN-inducible transcripts, whereas the alternative exon 1B and 1C structures at the 5′-termini of constitutively expressed ADAR1 transcripts

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both lack AUGs; translation initiation of the constitutively expressed 931 amino acid protein (p110) begins at the in-frame AUG296 present in exon 2 (George and Samuel, 1999; Valente and Nishikura, 2005; Toth et al., 2006). A second ADAR gene, *ADAR2*, maps to human chromosome 21q22 and encodes an ~80-kDa RNA adenosine deaminase that is a nuclear protein. ADAR2 is not regulated by IFN (Melcher et al., 1996; Villard et al., 1997; Toth et al., 2006).

Both p150 and p110 are active ADAR1 deaminases (Toth et al., 2006). The p150 and p110 proteins possess, in addition to the deaminase catalytic domain present in their C-terminal region, three copies of a dsRNA-binding motif in the central region that is homologous to the dsRNA-binding motif first discovered in the RNA-dependent protein kinase PKR (Kim et al., 1994; O'Connell et al., 1995; Patterson and Samuel, 1995; Liu and Samuel, 1996). The IFN-inducible p150 protein is N-terminally extended compared to p110, and possesses two copies of a Z-DNA binding motif (Patterson and Samuel, 1995; Athanasiadis et al., 2005). Two viral interferon resistance gene products that antagonize PKR kinase activity, the vaccinia virus E3L protein (Chang and Jacobs, 1993; Zhang et al., 2008) and the adenovirus VAI RNA (Kitajewski et al., 1986), also antagonize ADAR1 deaminase activity (Lei et al., 1998; Liu et al., 2001).

PKR is well established as an IFN-inducible antiviral protein and an important component of the IFN innate antiviral defense system (Haller et al., 2006; Toth et al., 2006; Sadler and Williams, 2008). PKR is a cytoplasmic RNA sensor; dsRNA and structured single-stranded RNAs are bound by PKR and mediate autophosphorylation and activation of PKR (Samuel, 1993). The best characterized substrate of PKR is the α subunit of translation initiation factor eIF-2, which when phosphorylated on serine 51, leads to an inhibition of protein synthesis in IFN-treated infected cells (Samuel, 1979; García et al., 2006; Sadler and Williams, 2008). Among IFN sensitive viruses is vesicular stomatitis virus (VSV), a negative-strand RNA virus widely used in IFN antiviral studies (Samuel, 2001; Haller et al., 2006; Randall and Goodbourn, 2008). Type I IFNs induce an antiviral state against VSV characterized by an inhibition of VSV protein production by a mechanism in which the PKR kinase plays an important role (Masters and Samuel, 1983; Balachandran et al., 2000; García et al., 2006).

Because PKR is activated by dsRNA, and because ADAR1 acts on dsRNA, to test whether ADAR1 affects PKR activation and VSV growth, cells in which >85% of both basal and IFN-inducible ADAR1 protein expression was stably silenced using a short hairpin RNA interference approach were examined. By comparing wild-type parental HeLa cells and control knockdown HeLa cells that are ADAR1-sufficient with the ADAR1-deficient knockdown cells, we found that ADAR1 loss did not affect the growth of VSV in the absence of IFN treatment. Likewise, overexpression of either ADAR1 or ADAR2 did not affect VSV single-cycle growth in 293 cells. However, in IFN-treated cells, the loss of ADAR1 led to enhanced activation of PKR and reduced yields of VSV.

Results

Overexpression of ADAR does not affect vesicular stomatitis virus growth

The single-cycle growth of VSV was examined in HEK 293 cell lines engineered to overexpress either ADAR1 p150 or ADAR2 protein to levels ~7 to 8 fold higher than that of parental 293 cells (Maas et al., 2001). As shown in Table 1, neither ADAR1 nor ADAR2 overexpression significantly affected VSV multiplication compared to parental 293 cells. The single-cycle yields at 24 h after infection of ADAR1 or ADAR2 overexpressing cells (2800 and 3200 PFU/cell, respectively) differed by only ~two-fold from that of the control parental 293 cells (6500 PFU/cell). Because it was possible that multiple rounds of replication might be necessary in order to accumulate sufficient mutations resulting from A-to-I editing to cause a measurable effect on the yield of infectious progeny, we then passed the virus for ten rounds of growth in 293 cells that stably overexpressed ADAR1 or ADAR2 as well as in control 293 cells. In all three 293 cell lines, the yield of infectious progeny remained relatively unchanged for three passages, decreased during passages 4 and 5, and then increased during passage 6 and began cycling (data not shown), a phenomenon described earlier for VSV (Huang and Baltimore, 1970; Palma and Huang, 1974).

Table 1
Stable overexpression of ADAR1 p150 or ADAR2 does not affect the single-cycle yield of vesicular stomatitis virus[a].

Cell	Time after infection (hours)		
	4	6	24
	(PFU/cell)	(PFU/cell)	(PFU/cell)
HEK293	220	2200	5600
293-ADAR1wt	420	1000	2800
293-ADAR2wt	520	1600	3200

[a] Virus yields were measured with the Indiana serotype of VSV in parental HEK 293 cells (HEK 293), and HEK 293 cell clones stably overexpressing either recombinant ADAR1 p150 (293-ADAR1wt) or ADAR2 (293-ADAR2wt) wild-type protein. Cells were harvested at the indicated times after infection, and virus yields determined by plaque titration on mouse fibroblast L cells.

Stable knockdown of cytoplasmic and nuclear ADAR1 proteins

Because overexpression of ADAR1 did not significantly affect VSV multiplication, we next wished to test the effect of ADAR1 p110 and p150 deficiency on virus growth. The HeLa cell clonal line 132 was examined in which a short hairpin-based RNA interference strategy was utilized to silence ADAR1 gene expression (Toth et al., 2009). As measured by Western analysis of whole-cell extracts (Fig. 1A), the

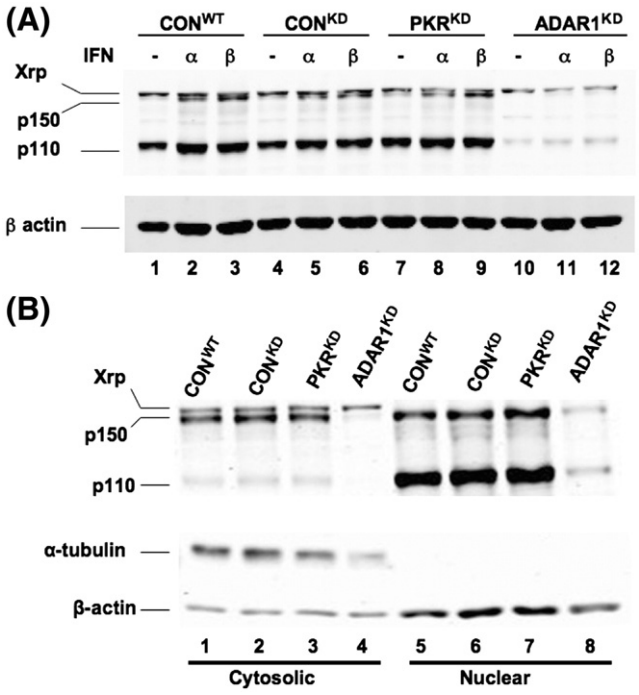

Fig. 1. Stable knockdown of ADAR1 proteins. Western immunoblot analyses comparing ADAR1 expression in wild-type parental (CON^{WT}), ADAR1 stable knockdown ($ADAR1^{KD}$), puromycin-resistant control (CON^{KD}) and PKR stable knockdown (PKR^{KD}) HeLa cell clones. The large (150 kDa) and the small (110 kDa) size forms of ADAR1 are designated p150 and p110, respectively. Xrp, a non-specific cross-reacting protein migrating just above p150. (A) Whole-cell extract analysis: cells were mock treated or treated with either 1000 units/ml of IFN-αA/D or IFN-β for 24 h. Whole-cell extract protein (10 μg) was analyzed in each lane on SDS–10% PAGE. (B) Analysis of fractionated cell lysates. Cytosolic and nuclear extract fractions were prepared from cells that had been treated with IFN-β 24 h and analyzed on SDS–7% PAGE. Membranes were probed using a polyclonal antibody against human ADAR1 and monoclonal antibodies against β-actin and α-tubulin as loading and fractionation controls (Ahn et al., 2004).

ADAR1 deficient clone 132 (designated ADAR1^{KD}) (lanes 10–12) showed <15% of p110 ADAR1 protein, and <10% of p150 ADAR1 protein, compared to the levels seen in ADAR1-sufficient HeLa cell lines. Three different ADAR1-sufficient lines were used as controls: the parental HeLa cells (CON^{WT}), a negative control puromycin-resistant clone shRNA pSUPER.retro.puro (CON^{KD}), and the HeLa cell clone (designated PKR^{KD}) which is stably knocked down for PKR expression (Zhang and Samuel, 2007). While both IFN-α and IFN-β induced p150 ADAR1 in the CON^{WT}, CON^{KD} and PKR^{KD} cells, the level of p150 ADAR1 remained very low in the ADAR1^{KD} cells even after treatment with type I IFN α or β (Fig. 1A).

When separate cytoplasmic and nuclear subcellular fractions were analyzed from IFN-β treated cells (Fig. 1B), the p110 ADAR1 protein was found predominantly if not exclusively in the nuclear fraction, whereas the p150 protein was found in both the cytoplasmic and nuclear fractions as originally described (Patterson and Samuel, 1995). The stable shRNAi knockdown of ADAR1 reduced the level of p150 ADAR1 to nearly an undetectable level, both in the cytoplasmic and the nuclear subcellular fractions. The protein cross-reacting with ADAR1 antiserum (Xrp) that migrated slightly more slowly than p150 on SDS–PAGE was a cytoplasmic protein, and was not reduced by the ADAR1 knockdown (Fig. 1B).

The ADAR1 targeting region for the knockdown is located in the ADAR1 3′-UTR corresponding to nt positions 4782–4802 (Toth et al., 2009), with the A of AUG1 taken as +1 in the ADAR1 sequence U18121 (Patterson and Samuel, 1995). The finding that both the inducible p150 and the constitutive p110 ADAR1 proteins were stably knocked down by the single shRNAi that targeted the 3′-UTR sequence is consistent with the conclusion that structures of the inducible transcript encoding p150 and the constitutively expressed transcript encoding p110 differ only in their alternative exons 1 and 7, but otherwise are identical including the 3′-UTR (George and Samuel, 1999; George et al., 2005). Finally, in the PKR^{KD} cells, the level of ADAR1 was comparable to the control CON^{WT} and CON^{KD} cells (Figs. 1A, B).

Effect of ADAR1 deficiency on cell growth

Two tests were performed to assess whether the stable knockdown of ADAR1 affected cell growth in culture, either in the absence or presence of treatment with IFN-β. First, as measured by direct cell count during subconfluent growth in monolayer culture, there was no significant difference in growth rate between the CON^{WT} and CON^{KD} cells either untreated or treated with IFN-β and the ADAR1^{KD} cells in the absence of IFN treatment. By contrast, over a 48 h period in the presence of IFN-β, for ADAR1^{KD} cells there was a significant ($p<0.001$student t-test) reduction in cell number, ~60%, compared to untreated cultures (data not shown).

The cell cycle distribution was next examined. As determined by propidium iodide staining to measure DNA content, the cell cycle distribution during subconfluent growth in monolayer culture was comparable for the ADAR1^{KD} and the ADAR1-sufficient CON^{KD} cells. About 45–50% of the cells were in G_0/G_1, about 20% of the cells were in S, and about 30% in G_2/M. As a control, thymidine-block CON^{KD} cells showed a substantially increased number of cells in S phase and decreased number in G_0/G_1.

Effect of ADAR1 deficiency on vesicular stomatitis virus growth and interferon sensitivity

Little is known regarding the effect that loss of ADAR1 has on virus replication and the antiviral action of interferon in human cells. To begin to address this question, we first tested the ability of VSV, a widely utilized virus in IFN studies, to multiply in ADAR1^{KD} compared to ADAR1-sufficient (CON^{WT}, CON^{KD}) cells. As shown in Table 2, VSV yields in the absence of IFN treatment were comparable in the ADAR1^{KD} cells and the two ADAR1-sufficient HeLa cell lines. However, treatment of cells with IFN consistently reduced the yield of infectious VSV to a lower level in the ADAR1^{KD} cells compared to ADAR1-sufficient cells (Table 2). The yield of infectious VSV was reduced about 200- to 300-fold by IFN-β treatment of the parental CON^{WT} and control CON^{KD} ADAR1-sufficient lines. By contrast, the antiviral state was enhanced in ADAR1^{KD} cells. Treatment of ADAR1^{KD} cells with IFN-β gave a reduction of about 2000-fold in VSV yield, typically ~1 $\log_{10}$ further inhibition than that seen in either ADAR1-sufficient cell line. In four independent experiments, the VSV yield in IFN-treated ADAR1^{KD} cells was about 16.5-fold less than in IFN-treated ADAR1-sufficient control cells. These results indicate that ADAR1 has a negative or suppressing effect on the establishment of an antiviral state against VSV.

Table 2
Antiviral activity of interferon measured with vesicular stomatitis virus in cells stably deficient for ADAR1 compared to ADAR1-sufficient cells[a].

Cell	Virus yield		
	Untreated (PFU/ml)	IFN-treated (PFU/ml)	Reduction (fold)
CON^{WT}	5.3×10^7	5.3×10^5	189
CON^{KD}	3.5×10^7	1.3×10^5	276
ADAR1^{KD}	3.0×10^7	1.4×10^4	2072

[a] Virus yields were measured with the Indiana serotype of VSV in wild-type parental (CON^{WT}), puromycin-resistant control (CON^{KD}), and ADAR1 stable knockdown (ADAR1^{KD}) HeLa cell clones either untreated or treated with 1000 units/ml IFN-® prior to infection as described under Materials and methods.

Stable knockdown of ADAR1 does not affect the expression level of PKR or STAT1 proteins

The basal and inducible expression levels of two other type I IFN-regulated proteins, STAT1 (Fig. 2A) and PKR (Fig. 2B), were assessed by Western analysis of ADAR1-deficient compared to ADAR1-sufficient cells. As shown in Fig. 2, neither PKR nor STAT1 steady-state protein level was affected by the knockdown of ADAR1. Likewise, the levels of β-actin and α-tubulin were unaffected in the

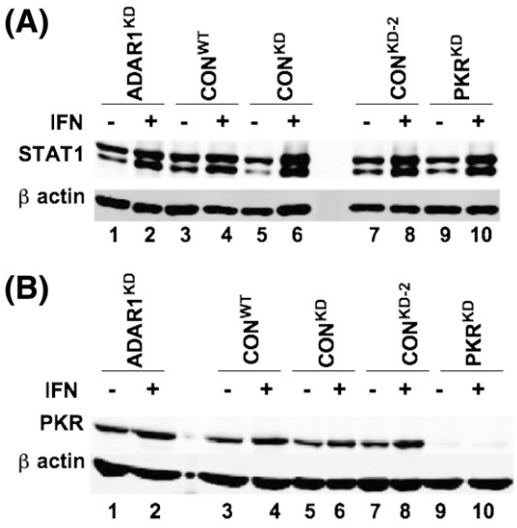

Fig. 2. Steady-state levels of PKR and STAT1 proteins in HeLa clones. Western immunoblot analyses comparing PKR and STAT1 expression in ADAR1 stable knockdown (ADAR1^{KD}), wild-type parental (CON^{WT}), puromycin-resistant control (CON^{KD}, CON^{KD2}) and PKR stable knockdown (PKR^{KD}) HeLa cell clones. Cells were left untreated or treated with IFN-αA/D for 24 h, whole-cell extracts were prepared, fractionated on SDS–10% PAGE (20 μg protein per lane), and membranes probed with antibodies against (A) STAT1 and (B) PKR. β-actin was used as a loading control.

ADAR1^{KD} cells (Figs. 1 and 2). The levels of PKR in the three PKR-sufficient cell lines, CON^{WT}, CON^{KD} and ADAR1^{KD}, were comparable, whereas the PKR^{KD} stable knockdown clone had <5% of the PKR protein level seen in the PKR-sufficient parental cells as previously described (Zhang and Samuel, 2007).

ADAR1 deficiency leads to enhanced PKR activation

Because the antiviral activity of IFN against VSV was enhanced in the ADAR1-deficient cells (Table 2), and because ADAR1 binds dsRNA (Liu and Samuel, 1996; Toth et al., 2006) and furthermore because ADAR1 was discovered based on its dsRNA-destablizing activity (Bass and Weintraub, 1988; Rebagliati and Melton, 1987), we considered the possibility that ADAR1 acts in part through modulation of PKR kinase activation which is mediated by RNA with double-stranded character (Toth et al., 2006; Sadler and Williams, 2008). To test this possibility, the effect of ADAR1 knockdown on PKR activation status measured by phosphorylation at threonine 446 was determined in VSV-infected cells that had been treated with IFN-β or not (Fig. 3). The human PKR protein is autosphosphorylated on multiple serine and threonine residues, one of which is T446, and these phosphorylations cause a decrease in electrophoretic mobility of PKR (Thomis and Samuel, 1993; Samuel, 2001).

In the cells stably knocked down for ADAR1 (ADAR1^{KD}) and treated with IFN, PKR phosphorylation at T446 was enhanced compared to the ADAR1-sufficient CON^{KD} cells (Fig. 3, lane 19 compared to lane 11). Transient knockdown of ADAR1 in the CON^{KD} cells by transfection with chemically synthesized siRNA against ADAR1 also led to increased PKR phosphorylation (lane 13) compared to an siRNA against luciferase (Luc) as a control (lane 12). Cells stably knocked down for PKR expression (PKR^{KD}), and either subsequently transiently knocked down for ADAR1 or not, did not show a detectable PKR T446 phosphorylation signal (Fig. 3, lanes 15–18). The level of PKR protein was comparable in the CON^{KD} and ADAR1^{KD} cells (Fig. 3, lanes 1–2, 9–10) in the absence of IFN treatment as earlier shown (Fig. 2B). Surprisingly, the enhanced PKR phosphorylation at T446 observed in VSV-infected cells in which ADAR1 was either stably (lanes 19, 20) or transiently (lane 13) knocked down was dependent upon treatment with IFN; parallel cultures that were left untreated (lanes 3, 9, 10) did not show the enhanced PKR activation irrespective of ADAR1 knockdown, either stable or transient (Fig. 3).

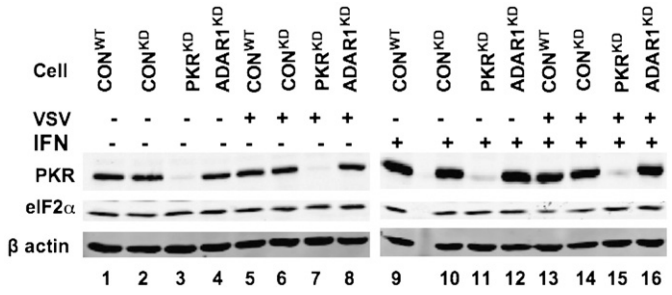

Fig. 4. Vesicular stomatitis virus infection does not alter the steady-state level of PKR or eIF-2α proteins in ADAR1^{KD} cells. Western immunoblot analyses comparing PKR and eIF-2α expression in wild-type parental (CON^{WT}), puromycin-resistant control (CON^{KD}), PKR stable knockdown (PKR^{KD}), and ADAR1 stable knockdown (ADAR1^{KD}) HeLa cell clones, either mock treated (–, lanes 1–8) or treated with 1000 units/ml IFN-β for 16 h (+, lanes 9–16) prior to infection with VSV (+) or were left uninfected (–). Whole-cell extracts were prepared at 8 h after infection, fractionated on SDS–7% PAGE (20 μg protein per lane), and analyzed by western immunoblot utilizing antibodies against PKR, eIF-2α, and β-actin.

We next tested whether VSV infection altered the steady-state protein level of either PKR or eIF-2α in ADAR1^{KD} cells compared to ADAR1-sufficient cells. As shown in Fig. 4, the level of PKR protein was comparable in CON^{WT}, CON^{KD} and ADAR1^{KD} cells, both uninfected cells (lanes 1–4) and cells infected for 8 h (lanes 5–8), all in the absence of IFN treatment. IFN treatment prior to infection led to comparably increased levels of PKR protein in all three of these PKR-sufficient cell lines, but did not alter the level of the eIF-2α factor (Fig. 4). In the PKR^{KD} cells, the PKR protein was marginally detectable even after IFN treatment (Fig. 4, lanes 3, 7, 11, 15) as previously described (Zhang et al., 2008). However, serine 51 phosphorylation of eIF-2α was increased in VSV-infected IFN-treated ADAR1^{KD} cells (Fig. 5, lane 4) compared to the ADAR1-sufficient CON^{WT} and CON^{KD} cells where the eIF-2αS51 phosphorylation was comparable to PKR^{KD} cells (Fig. 5, lanes 1–3). These results suggest that the depletion of ADAR1 leads to enhanced activation of PKR as measured by T446 phosphorylation in VSV-infected cells, and that the increased PKR phosphorylation is dependent upon IFN treatment. Furthermore, the elevated phosphorylation of PKR seen in the ADAR1-deficient IFN-treated VSV-infected cells (Fig. 3) correlates with the enhanced antiviral activity (further reduced yields of VSV) found in the ADAR1^{KD} cells compared to ADAR1-sufficent cells (Table 2) and increased eIF-2α phosphorylation (Fig. 5).

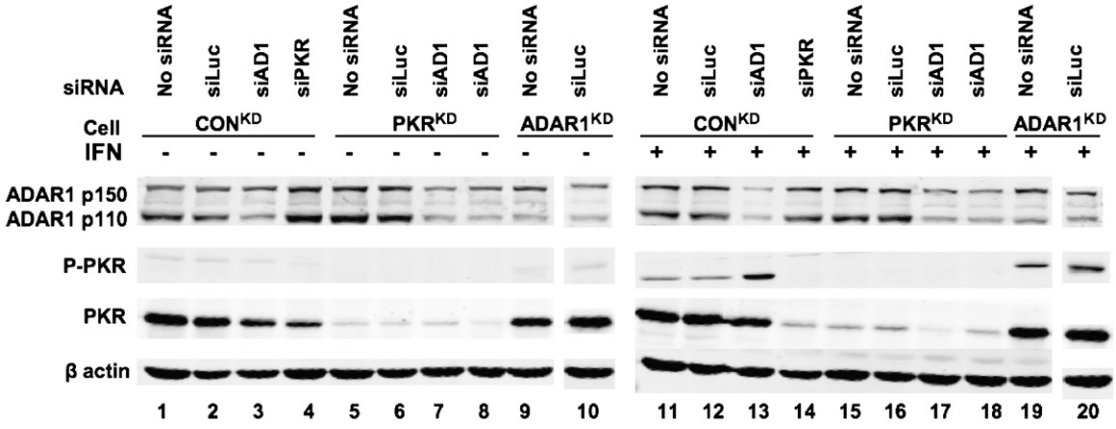

Fig. 3. Effect of ADAR1 knockdown either stably or transiently on activation of PKR kinase. ADAR1 stable knockdown (ADAR1^{KD}), PKR stable knockdown (PKR^{KD}) and puromycin-resistant control (CON^{KD}) HeLa cell clones were subsequently transiently knocked down utilizing chemically synthesized siRNAs against luciferase (siLuc) as a control, ADAR1 (siAD1), or PKR (siPKR) as described under Materials and methods. Following transient knockdown, the cells in monolayer culture were either mock treated (–, lanes 1–10) or treated with 1000 units/ml IFN-β for 16 h (+, lanes 11–20) prior to infection with VSV. Whole-cell extracts were prepared at 8 h after infection, fractionated on SDS–10% PAGE (30 μg protein per lane), and analyzed by western immunoblot analysis utilizing antibodies against PKR and phospho-threonine 446 PKR (P-PKR). β-actin was used as a loading control.

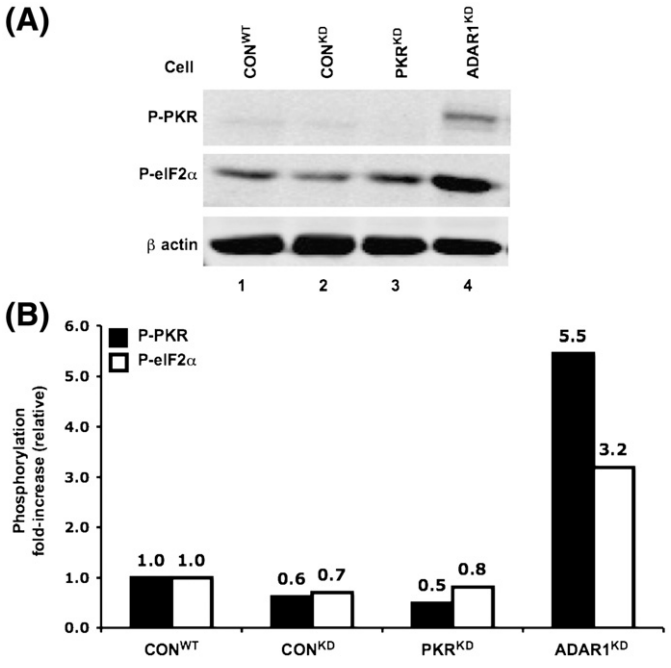

Fig. 5. ADAR1 knockdown leads to increased phosphorylation of protein synthesis initiation factor eIF-2α. Wild-type parental (CON^{WT}), puromycin-resistant control (CON^{KD}), PKR stable knockdown (PKR^{KD}), and ADAR1 stable knockdown ($ADAR1^{KD}$) HeLa cell clones were treated with 1000 units/ml IFN-β for 16 h, and then infected with VSV for 8 h. Whole-cell extracts were prepared, fractionated on SDS–7% PAGE (30 μg protein per lane), and analyzed by western immunoblot analysis for the T446 phosphorylation of PKR (P-PKR), S51 phosphorylation of eIF-2α (P-eIF2α), and β-actin.

Discussion

In this study we used human cell lines in which >85% of ADAR1 was stably depleted by an RNA interference strategy, and cell lines stably overexpressing ADAR1 or ADAR2, to investigate the roles of ADAR1 in both cell growth and virus multiplication. Several important points emerge from our findings.

First, the substantial reduction of ADAR1 proteins, both basal p110 and IFN-inducible p150, achieved in the $ADAR1^{KD}$ cells did not significantly affect either cell growth or vesicular stomatitis virus replication in culture in the absence of IFN treatment. VSV grew comparably well in $ADAR1^{KD}$ and ADAR1-sufficient cells when left untreated with IFN. Likewise, overexpression of either ADAR1 p150 or ADAR2 did not affect VSV single-cycle virus yields. The $ADAR1^{KD}$ cells, however, were more sensitive to the antiviral actions of type I IFN than the parental CON^{WT} and the knockdown control CON^{KD} ADAR1-sufficient cells. What molecular mechanism might account for the further reduction in VSV growth seen in the IFN-treated $ADAR1^{KD}$ cells compared to CON^{WT} or CON^{KD} ADAR1-sufficient cells?

PKR was found to be an important component of the IFN-mediated resistance to VSV infection in earlier studies using either the catalytic domain disrupted *Pkr* knockout mice and derived *Pkr* null MEF cells (Stojdl et al., 2000) or human HeLa cells in which PKR was stably knocked down (PKR^{KD}) by a short hairpin RNA interference strategy (Zhang and Samuel, 2007). In the absence of IFN treatment, VSV yields are comparable in PKR^{KD} and CON^{KD} HeLa cells, but in IFN-treated PKR^{KD} cells the yield reduction is modestly different, only ~10–30 fold in PKR^{KD} compared to ~100–300 fold in CON^{KD} cells (Zhang and Samuel, 2007), similar to the reduction observed herein in CON^{KD} cells (Table 2). We therefore considered the possibility that ADAR1 may exert a suppressive modulatory effect on PKR that is relieved in the ADAR1 knockdown cells. Indeed, ADAR1 binds dsRNA and through A-to-I RNA editing destabilizes dsRNA structures (Liu and Samuel, 1996; Toth et al., 2006), the very property by which ADARs were originally discovered (Rebagliati and Melton, 1987; Bass and Weintraub, 1988). By contrast, PKR is activated by RNA with double-stranded character (Samuel, 1979). We found that the activation of PKR as measured by T446 phosphorylation was increased in IFN-treated $ADAR1^{KD}$ cells, and furthermore, that this increased activation of PKR correlated with a reduced virus growth in IFN-treated $ADAR1^{KD}$ cells compared to ADAR1-sufficient cells. Although transient overexpression of ADAR1 also has been reported to enhance VSV replication and to impair phosphorylation of PKR and eIF-2α (Nie et al., 2007), we were unable to confirm this observation using HEK293 cells stably overexpressing either ADAR1 p150 or ADAR2. Curiously the reported proviral effect of ADAR1 overexpression was dependent upon the N-terminal RNA-binding domains but not the catalytic deaminase domain of ADAR1 (Nie et al., 2007; Clerzius et al., 2009). However, it is known that such an RNA-binding domain dependent response is not unique to ADAR1. Other dsRNA-binding proteins, as illustrated by vaccinia virus E3L, reovirus σ3 and TAR RNA-binding protein, also are known to antagonize PKR activation and enhance virus growth (Haller et al., 2006; Randall and Goodbourn, 2008; Samuel, 2001; Zhang et al., 2008).

Genetic disruption of *Adar1* in mice is embryonic lethal at E11.5–12.5 and occurs with liver disintegration (Hartner et al., 2004; Wang et al., 2004). ADAR1 is essential for the maintenance of hematopoietic stem cells, and genetic loss of ADAR1 leads to up regulation of type I and type II IFN-inducible transcripts in hematopoietic stem cells but not other cells (Hartner et al., 2009). Our results are in agreement with these observations. We found that ADAR1 knockdown in HeLa cells did not affect either PKR or STAT1 induction by type I IFN, and Hartner et al. (2009) found that ADAR1 genetic deficiency likewise did not affect STAT1 transcript levels in macrophages or neutrophils. These observations taken together suggest that ADAR1 likely mediates effects on IFN signaling, and possibly cell growth, in a cell-specific manner. We have described a PKR-dependent induction of IFN-β and apoptosis in cells transfected with dsRNA (McAllister and Samuel, 2009), and PKR transcripts are reported to be up regulated in ADAR1-deficient hematopoietic stem cells (Hartner et al., 2009). Conceivably such an ADAR1-mediated modulation of PKR induction and activation contributes to the apoptosis and early embryonic lethality seen in mice (Hartner et al., 2004, 2009; Wang et al., 2004).

One surprising observation in our study was that type I IFN treatment alone was sufficient to mediate enhanced activation of PKR in ADAR1-deficient cells, both in cells stably knocked down or cells transiently knocked down for ADAR1 protein. VSV infection was not required in addition to the IFN-β treatment. An intriguing possibility is that the IFN treatment leads to induction of cellular transcripts with the capacity to activate PKR, and that these transcripts also are substrates for modification by ADAR1. The best characterized ADAR1 cellular substrate RNAs with double-stranded character, predominantly neurotransmitter receptors and channels, are not known to be IFN-regulated (Samuel, 2001), although computational analyses have identified a number of additional candidate cellular RNA targets for A-to-I editing (Toth et al., 2006). Possibly one or more of these are IFN-inducible, and capable of PKR activation.

Type I IFN treatment induces expression of both PKR (Tanaka and Samuel, 1994; Kuhen and Samuel, 1997) and the p150 size form of ADAR1 (Patterson and Samuel, 1995; George and Samuel, 1999; 2008). p150 ADAR1, like PKR, is found in the cytoplasm of IFN-treated cells (Toth et al., 2006). It is tempting to speculate that one function of the IFN-inducible ADAR1 dsRNA-editing enzyme is to suppress RNA-dependent activation of the IFN-inducible PKR kinase. That ADAR1 has a positive effect on virus replication through suppression of PKR, as we observe for VSV herein under conditions of IFN treatment, also has been observed with two other RNA viruses with vastly different replication strategies, human immunodeficiency virus (Clerzius et al., 2009; Doria et al., 2009) and measles virus (Toth et al., 2009). Whether the counter-balancing effect of one IFN-inducible enzyme (ADAR1) on another (PKR) is significant in PKR-modulated biologic

responses in addition to virus replication, for example apoptosis and signaling, remains to be established.

Materials and methods

Cells and viruses

HeLa cells, 293 human embryonic kidney (HEK) cells and mouse fibroblast L cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (HyClone), 100 μg/ml of penicillin, and 100 units/ml of streptomycin (GIBCO/Invitrogen) as previously described (Zhang and Samuel, 2007). HeLa cells stably knocked down for either PKR ("PKR^{KD}") (Zhang and Samuel, 2007; Zhang et al., 2008) or ADAR1 ("$ADAR1^{KD}$") (Toth et al., 2009) established using a short hairpin RNA interference strategy, and drug-resistant knockdown controls cells ("CON^{KD}"), were maintained in the above-named maintenance medium containing 1 μg/ml puromycin (Sigma). The HeLa cell parental line was sometimes used as an additional control and is designated as CON^{WT}. HEK 293 cell lines stably overexpressing ADAR1 or ADAR2 (Maas et al., 2001) were generously provided by S. Maas and A. Rich (MIT, Cambridge, MA). The Indiana serotype of vesicular stomatitis virus (VSV) was as previously described (Samuel and Knutson, 1981; Zhang and Samuel, 2007). Interferon treatment was with 1000 units/ml of recombinant IFN, either IFN-αA/D (PBL InterferonSource) or IFN-β (Toray Industries), for 16 h unless otherwise as indicated.

Transient knockdown

Transient knockdown of either PKR or ADAR1 in human cells was carried out using chemically synthesized siRNAs. The siRNAs were obtained from Dharmacon with dTdT overhangs and the following sequences: PKR, sense strand 5′-GCAGGGAGUAGUACUUAAAATT-3′ (Zhang and Samuel, 2007) and ADAR1, sense strand 5′-CGCAGAGUUCCUCACCUGUATT-3′ (Jayan and Casey, 2002). Transfection of siRNAs was performed with lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Two cycles of transient transfection were necessary to achieve maximal transient knockdown of ADAR1 in the HeLa cells stably knocked down for PKR, and conversely transient knockdown of PKR in the cells stably knocked down for ADAR1. Briefly, nearly confluent cells were transfected with siRNA targeting ADAR1 on day 1, then on day 2 the cells were split and seeded at 60% confluence. On day 4 the cells were transfected again with the appropriate siRNA and then tested for phenotype 36 to 48 h later. Interferon treatment was with 1,000 units/ml of either IFN-αA/D or IFN-β indicated.

Virus infections and growth assays

$ADAR1^{KD}$, CON^{KD}, and/or CON^{WT} cells were infected with vesicular stomatitis virus at an MOI of 0.5 or 10 as indicated. Virus adsorption in monolayer culture was carried out in Puck's saline A modified to contain 10 mM $MgCl_2$ and 1% (v/v) fetal bovine serum. After 1 h the inoculum was removed, the monolayers rinsed twice, and the cultures then incubated in maintenance medium with 5% (v/v) serum. Infected cultures were harvested at the indicated time postinfection by scraping the infected cells into the medium. Virus yields were determined by plaque titration, using mouse L cells (Samuel and Knutson, 1981; Zhang and Samuel, 2007).

Western immunoblot analyses

Whole-cell extracts, and cytosolic and nuclear fractions, were prepared in the presence of 1 mM phenylmethylsulfonyl fluoride and 1% (v/v) protease inhibitor mixture (Sigma) as previously described (Das et al., 2006; Zhang and Samuel, 2007). For analysis of PKR and eIF-2α phosphorylation status, lysis buffers were supplemented 1% (v/v) phosphatase inhibitor cocktail (Sigma). Protein concentration of the extract fractions was determined by the Bradford method. Proteins were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using either 7% or 10% acrylamide gels as indicated, transferred to nitrocellulose, and the membranes blocked in Tris-buffered saline containing either 5% (w/v) bovine serum albumin (for detection of phospho proteins) or skim milk (for detection of other proteins). Rabbit polyclonal antibody against ADAR1 (K88#2) was previously described (Patterson and Samuel, 1995). Rabbit polyclonal antibodies from the indicated sources were used to detect human PKR (Santa Cruz), eIF-2α (Cell Signaling), eIF-2α phosphorylated at Ser51 (Cell Signaling), and STAT-1 (Santa Cruz). Either rabbit polyclonal (Santa Cruz) or monoclonal (Epitomics) antibody was used to detect PKR phosphorylated at Thr446. Mouse monoclonal antibodies were used to detect FLAG epitope-tagged ADAR (Sigma), β-actin (Sigma), and α-tubulin (Sigma). Blot detection was performed with IRDye 800CW-conjugated anti-rabbit IgG or IRDye 680-conjugated anti-mouse IgG secondary antibody according to the manufacturer's protocols (Li-Cor Biosciences). Immunoreactive bands were visualized using a Li-Cor Odyssey infrared imaging system.

Cell cycle analyses

HeLa cell clones, knockdown or control as indicated, were cultured in maintenance medium and either left untreated or treated with IFN-β at 1000 units/ml for 24 h. Cells were analyzed at ~70% confluence in monolayer. For cell cycle analyses, cells were fixed with 70% ethanol overnight, rinsed with PBS, and then treated with RNase A (200 μg/ml) for 30 min at 37°C. After staining with propidium iodide (10 μg/ml, Sigma), the percentages of cells in G_0/G_1, S, and G_2/M were determined using a Guava Technologies Easy Cyte instrument and CytoSoft software. Double thymidine block-treated samples (Sansregret et al., 2006) were used as a positive control.

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